Application of molecular techniques in population genetic studies of cystic fibrosis in the Netherlands

de Vries, Hendrik Gerardus

Chapter 1

General introduction

1.1 Delineation of and research into cystic fibrosis

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder affecting Caucasian populations, with a generally quoted birth prevalence of one in 2500 [1].

Table 1. Historical perspectives of cystic fibrosis research.

1936	Report of a child with the clinical features of cystic fibrosis.	[2]
1938	First detailed description of the clinical features and pathology of CF: "Cystic fibrosis" of the pancreas.	[3]
1945	Recognition of the role of sticky mucus as a cause of many of the symptoms: "Mucoviscidosis".	[4]
1946	Suggestion of an autosomal recessive inheritance pattern for CF.	[5]
1953	Investigation of acute salt loss caused by excessive sweating in babies with CF, during a heat-wave.	[6]
1959	Development of the pilocarpine iontophoresis method for sweat testing.	[7]
1981	Description of altered electrical properties of CF respiratory epithelium associated with abnormalities of both sodium and chloride transport.	[8]
1983	Documentation of chloride impermeability of CF sweat gland ducts.	[9]
1985	Mapping of the CF gene to a small segment of the long arm of chromosome 7q, using restriction fragment length polymorphisms (RFLP).	[10]
1987	Linkage disequilibrium between the markers XV2C/KM19 and cystic fibrosis was observed.	[11]
1989	Cloning of the CF gene and description of a cDNA sequence.	[12]
1989	Identification of the ΔF508 mutation in approximately 70 percent of the mutations.	[13]
1991	Report of the total genomic sequence of the CFTR gene.	[14]
1994	Description of more then 500 different CF mutations.	[15]

The disease is characterized by abnormal secretions of the exocrine glands which cause chronic obstruction and infection of the respiratory tract, meconium ileus, pancreatic insufficiency, and elevated levels of sweat electrolytes. Rochholz (Almanac of Children's Songs and Games from Switzerland, 1857) described " The child will soon die whose brows tastes salty when kissed". Table 1 shows important data on the delineation of and research into CF during the past 60 years.

1.2 The CFTR gene and its mutations

The gene responsible for cystic fibrosis (CF) has been identified in 1989 [12,13,16]. It is located on the long arm of chromosome 7, region 7q31.2 [17]. The gene is approximately 230,000 kb in size and contains 27 exons [14]. The encoded mRNA is about 6.5 Kb long. The gene codes for the 1480 amino acid cystic fibrosis transmembrane conductance regulator (CFTR) protein, with a molecular mass of 168,138 dalton. CFTR is a cAMP-induced chloride channel [18-30], which is located in epithelial tissues, including pancreatic ductal cells, salivary glands, intestine, lung, testis and endometrium [31-34]. Defective regulation of the chloride channel, caused by different mutations in the CF gene, results in desiccation of secretions, increase in the viscosity of mucus and a decrease in mucociliary clearance. The primary sequence of CFTR suggests that it is a transmembrane protein which consists of five domains (Figure 1): two membrane-spanning domains, each composed of six putative transmembrane segments; two nucleotide-binding domains; and a unique regulatory (R) domain [35,36]. The membrane-spanning domains contribute to the formation of the Cl^- channel pore. The nucleotide-binding domains and the regulatory domain control the channel activity through an interaction with cytosolic nucleotides. Phosphorylation of the regulator domain by cAMP-dependent kinase is required for the channel to open [37-39]. In addition to this function, the complex expression pattern of the gene and the varied degree of the clinical manifestations in different organs and tissues suggest that CFTR may have other activities [40-45].

Thus far more then 500 presumed mutations have been identified in the CFTR gene in the past 5 years [15]. These mutations can be divided in missense- (42%), frameshift- (23%), nonsense- (16%), splice defect- (16%) and deletion mutations

(4%). The distribution of mutations over the gene is nonrandom, with the highest density in NBF1 (14.4%) and to a lesser extent in NBF2 (10.8%) (Figure 1). Most of the CF mutations have been identified among patients of Caucasian ancestry. The population variation of CF mutations in Europe has been well documented by the Cystic Fibrosis Consortium [46].

Figure 2 shows four different mechanisms by which CF mutations disrupt the CFTR function [36]. **Class 1 mutations:** Mutations (G542X, 621+G->T, etc) that cause defective protein production by premature termination signals as a consequence of affected splice sites, frameshifts or nonsense mutations [35,47,48]. These mutations result in a shortage of CFTR Cl^- channels in affected epithelia since little or no full-length protein is produced. **Class 2 mutations:** Several mutant forms of CFTR fail to be transported correctly, which results in defective protein processing. Experiments with other proteins suggest that class 2 mutations (ΔF508, S559T, N1303K, etc) cause an incorrect folding of CFTR. Cellular mechanisms recognise mutant CFTR as abnormal and mark the protein for degradation

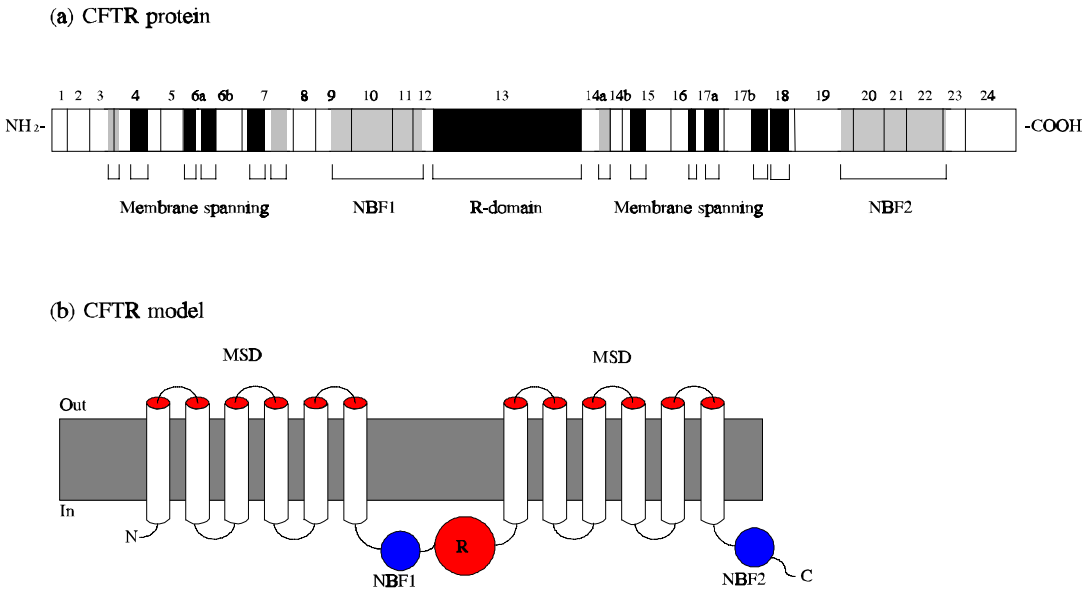

Figure 1. CFTR contains 27 exons and consists of five domains: two membrane-spanning domains (MSD), two nucleotide-binding domains (NBF1 and 2), and a regulatory (R) domain. (Adapted from Tsui and Buchwald)

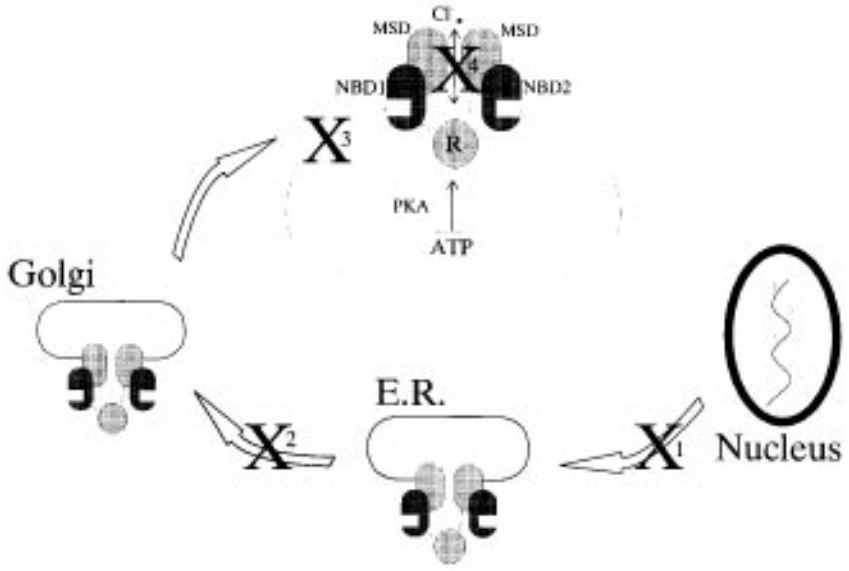

Figure 2. Biosynthesis and function of CFTR in an epithelial cell and action mechanisms of CF mutations. PKA, cAMP-dependent protein kinase; MSD, membrane-spanning domain; NBD, nucleotide-binding domain, R, regulatory domain; X1-4, Class 1-4 mutations; ER, endoplasmatic reticulum. (Adapted from Welsh and Smith)

nucleotide-binding domains (G551D, G551S, A455E, etc). [49,50]. **Class 3 mutations:** Defective regulation by mutations in the NBDs. Intracellular ATP regulates the opening of CFTR Cl^- channels through direct interaction with the nucleotide binding domains. Mutations in these domains, therefore, can alter the channel function. **Class 4 mutations:** Mutations (R117H, R334W, etc) that have been identified in the membrane spanning domains, which are thought to contribute to the channel pore [51]. This results in the generation of less Cl^- current because the rate of ion flow is reduced.

It is difficult to predict the clinical phenotype from a certain genotype. First, there is a diversity in the clinical phenotype, even within the same genotype. Variability may result from environmental factors and genetic background. Secondly, clinical abnormalities are difficult to predict in compound heterozygotes. Thirdly, patients may bear more than one mutation within the same CFTR gene, of

which only one has been identified. The state of pancreatic function allows discrimination of different phenotypes. Class 1 and 2 mutations are associated with a severe pancreatic-insufficient phenotype, while class 3 and 4 mutations show a normal pancreas function.

Approximately 70% of the CF chromosomes harbour a three basepair deletion in CFTR, which removes the phenylalanine residue at amino acid 508 of the predicted CFTR polypeptide (ΔF508) [46]. The estimates of the age of the ΔF508 mutation vary from 3,000 to 52,000 years [52-55]. According to the latter study, in which microsatellites were used in different CF populations in Europe, the ΔF508 mutation arose at least 52,000 years ago in a population genetically distinct from the present European population. The mutation was introduced in Europe in different periods. The first spread was during the Palaeolithic period. The Basque population is believed to be a Palaeolithic population [56], and has a high relative frequency of ΔF508 (88%) [53]. Later introduction of ΔF508 in Central and Northern Europe (Neolithic period) increased the frequency of this mutation in these regions. The relative ΔF508 frequencies are shown in Figure 3 [46]. The highest frequency per country can be found in Denmark (0.88), while the frequency decreases in Southern and Eastern directions.

1.3 Possibilities for genetic modification

A. Mouse models

The mouse homolog of the human CFTR gene was cloned in 1991 [57]. The mouse protein is 78% identical to the human CFTR, with a high conservation in the transmembrane and nucleotide-binding domains. A mouse model for cystic fibrosis made by gene targeting was first described in 1992 [58]. The murine CFTR gene in embryonic stem cells was inactivated by targeted insertion of a neomycin gene. This resulted in a frame stop codon in exon 10. Numerous mutant mice have been produced using this strategy [59-63]. Almost all mutant CF/CF mice displayed many features common to young CF patients, including failure to thrive, meconium ileus, alteration of mucous and serous glands. This resulted in death due to intestinal obstruction before 40 days of age.

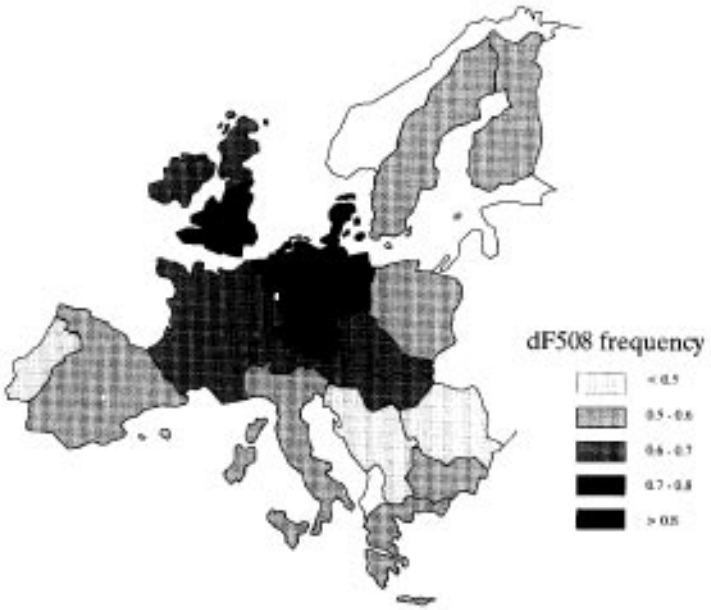

Figure 3. Relative frequency of the ΔF508 mutation in West and Central European populations. The dark shaded areas show a higher ΔF508 frequency compared with the light shaded regions. No data were available from Norway, Luxembourg, Albany and Eastern Europe. The high relative frequency of ΔF508 in the Basque population was averaged with the mutation frequency found in the rest of Spain.

One mutant mouse showed mild focal pulmonary atelectasis, consistent with presymptomatic lung disease, and mild dilatation of salivary ducts [60]. No evidence was obtained for abnormal pancreatic histology or defective reproductivity. The absence of pulmonary disorders in mice can be explained by the late onset of manifestations in humans. By alleviating the intestinal obstruction in CF mice, there is a possibility to observe more of the symptoms of human CF. However some of the differences between the human and mouse phenotypes may be related to tissue-specific patterns of expression of CFTR.

The availability of mouse models provides opportunities for the testing of new therapies and to study the intestinal pathophysiology of CF in greater detail. For example, to determine whether heterozygotes are more protected against exposure to cholera toxin (see 1.5).

B. Somatic gene therapy for cystic fibrosis

The clinical expressions of CF are very broad, although the manifestations in the airways account for more then 95% of the morbidity in CF patients [1]. It is therefore that gene transfer strategies are focused on delivery to the airways. A variety of gene transfer techniques have been used effectively in vitro and in animals: adenoviral vectors, liposome-mediated gene transfer, DNA-ligand complexes and adeno-associated viral (AAV) vectors [64]. The first two therapies are being used in phase I clinical trials involving CF patients.
Several studies indicate that even a low expression (10%) of the CF gene in airway epithelium may provide clinical benefit [65,66]. Healthy CF carriers will have half-normal levels of expression. There are some compound heterozygotes which show mild or normal phenotypes, while they have very low levels of expression [47,67]. This means that there is no necessity to correct all cells and even low levels of expression per cell may be beneficial.

The major goals for somatic gene therapy for cystic fibrosis are (1) to show that the expression of the CF gene in airways epithelium will alleviate lung disease in CF patients and (2) to achieve a lasting expression of the transferred gene.

Table 3. Major advantages and disadvantages of gene transfer systems.

	Advantages	Disadvantages
Adenoviral vectors	1. Highly efficient gene transfer 2. Transfection of many cell types	1. Transient nature of the expression 2. Pathogenicity of the wild type virus 3. No evidence for save and effective readministration.
DNA-liposome complexes	1. Standardized production of large amounts of vector 2. No risks of viruses 3. Readministrations with minimal host response	1. No persistent expression 2. Low gene expression
DNA ligand complexes	1. See DNA-liposome complexes	1. See DNA-liposome complexes 2. Large size of complexes may complicate escape from the vascular space 3. Peptides can induce immune responses
AAV vectors	1. Stable virus preparations 2. Lack of pathogenicity 3. High-efficiency integration at present	1. Possibility for immune responses 2. No adequate system to produce virus 3. Limitation of insert size to 4.8 kb

This can be performed through a single delivery with persistent expression or by use of a save and effective repetitive delivery system. The biological aspects of various gene transfer systems have been reviewed elsewhere [68,69]. The advantages and disadvantages of these strategies are summarized in table 3.

1.4 Cystic fibrosis prevalence at birth

Cystic fibrosis is the most common autosomal recessive disease in caucasian populations. Birth prevalences have been determined by surveys and range from 1 in 1700 to 1 in 40,000 in various European countries (Table 2). Thus far, estimates based on molecular screening for heterozygotes have not been described in European populations.

The estimates of the birth prevalences show a variability which as a whole or in part may be due to under- or overdiagnosis and under- or overreporting. Alternatively this variability may be due to mechanisms such as foundereffect and genetic drift.

Table 2. Birth prevalences of cystic fibrosis determined in European populations

Czechoslovakia	1 in 2600	1962 [70]
	1 in 2800	1964 [71]
	1 in 2700	1967 [72]
	1 in 3400	1970 [73]
	1 in 3300	1970 [74]
	1 in 5440	1972 [75]
Denmark	1 in 4500	1972 [76]
	1 in 4760	1988 [77]
England	1 in 3000	1967 [78]
	1 in 4100	1967 [79]
	1 in 2000	1968 [80]
	1 in 3000	1972 [81]
	1 in 2500	1988 [82]
Finland	1 in 40000	1972 [83]
France	1 in 3300	1961 [84]
	1 in 1700-2000	1971 [85]
	1 in 2000	1973 [86]
	1 in 1800	1974 [87]
	1 in 3200	1978 [88]
Germany	1 in 3300	1963 [89]
	1 in 3000	1968 [90]
	1 in 2000	1975 [91]

Table 2. (Continued)

Ireland	1 in	1800	1976 [92]
Italy	1 in	1100-3500	1973 [93]
	1 in	2700	1976 [94]
	1 in	2000	1985 [95]
Netherlands	1 in	3600	1975 [96]
N-Ireland	1 in	1700	1959 [Cited by Clarke, 97]
	1 in	1900	1979 [98]
Poland	1 in	900-2500	1970 [99]
Sweden	1 in	7700	1962 [100]
	1 in	4000	1970 [101]
	1 in	3500	1976 [102]
	1 in	2200-4500	1982 [103]
Switzerland	1 in	2900	1976 [104]
Turkey	1 in	3000	1973 [105]

1.5 What causes the high frequency of cystic fibrosis ?

The high birth prevalence of cystic fibrosis of about one in 2500 in the Caucasian population [1] has been discussed in many papers. A variety of mechanisms has been suggested to account for this high frequency: genetic drift [106], high mutation rate [107], meiotic drive and increased fertility of CF carriers [108], genetic heterogeneity [109], heterozygote advantage [109-113] and segregation distortion of CF alleles [108,114,115] . Heterozygote advantage is the most likely explanation. A positive selection of approximately 2% [113] would be sufficient to maintain the CF gene frequency at current levels.

The best understood example of heterozygote advantage remains sickle cell haemoglobin, and the protection it confers against malaria. In case of CF, abnormally functioning chloride channels could lead to increased protection against bacterial toxin-mediated diarrhoea. Some of the diseases which have been suggested as the balancing agent for CF are: influenza [116,117], syphilis [118], malaria [119], bubonic plaque [112] and cholera [119-122]. Gabriel et al. [123] showed reduced diarrhoea in the cystic fibrosis heterozygote mouse compared with the normal mouse after exposure to cholera toxin. This effect, however, has not been studied in human populations sofar.

1.6 Screening for cystic fibrosis carriers

The investigation of the validation of a mouthwash procedure and of the determination of the carrier prevalence in the Netherlands (Chapters 2 and 3) can partially be considered as preparatory studies for possible population-based screening programmes. Immediately upon the cloning of the CF gene in 1989, intensive discussions began regarding the feasibility and appropriateness of offering carrier testing to the general population. Wilfond and Fost [124] have reviewed many of the concerns in detail. The following arguments against population screening have been raised. (1) It is too expensive and not cost-effective. (2) It is not possible to identify all at-risk couples. (3) Families do not wish to avoid the disease. (4) Prevention through abortion is morally objectionable. (5) A cure for the disease is possible. (6) The personnel needed for genetic counselling are not available. (7) Many couples are left at slightly increased risk. (8) There is a risk of discrimination regarding insurance or employment. The following arguments in favour of carrier testing have been raised. (1) Testing will become less expensive and be cost-effective. (2) The majority of couples at-risk can be identified. (3) Many couples wish to avoid the disease through various reproductive options. (4) There is an obligation to inform the public about the availability of carrier testing. Pilot studies for CF testing can address some of this issues.

Investigations in different groups of people revealed a positive attitude toward carrier screening [125-128]. The American Society of Human Genetics and the National Institutes of Health stated that carrier screening would be desirable only if 90-95% of mutations can be detected. Present data suggest that the sensitivity can be well above 90% in some Northern European populations, for instance 98% in Belgium [129], 98% in a Celtic population in Brittany [130] and 99% in Wales [131]. The ΔF508 mutation can be detected by simple gel electrophoresis in material like blood samples, dried blood spots, mouthwashes, mouthbrushes or fetal cells. The application and validation of the mouthwash procedure for the DNA isolation out of buccal cells is described in Chapter 2. Most laboratories now test routinely for from 10 to over 30 common CF mutations using forward or reverse ASO methods [132] and the Amplification Refractory Mutation System [133]. Solid-phase methods based on ligation detection, single base sequencing and other tests offer great promise for the development of highly

automated procedures for the economical analysis of dozens of mutations simultaneously [134]. An economic two-step laboratory approach can be used for mutation detection [135]. In the first step only a few mutations will be determined and only the partners of identified carriers will be tested for additional mutations. The development of economic highly automated procedures for mutation analysis and the positive attitude toward carrier testing will strongly increase the demand for population based screening.

1.7 Identity By Descend (IBD) analysis

Haplotype sharing analysis is based on linkage disequilibrium of polymorphic loci with a disease gene. In previous investigations extensive linkage studies have been applied for the mapping of rare disease alleles. Although these studies provided valuable contributions toward the mapping of mutated genes, a few disadvantages can be observed using the traditional gene localization methods. (1) It is difficult to obtain adequate family material and large pedigrees. (2) After the selection of families all members should be examined concerning clinical features. (3) Hundreds of markers should be involved in a complete genome search. (4) Costs of material and manpower are high.

Identity by descent (IBD) analysis is an alternative association study method, in which unrelated patients in a founder population can be selected for gene mapping. Patients with identical genetic diseases are likely to share the disease gene and an area around that gene from a common ancestor. The size of the expected shared area depends on the number of meioses connecting the different persons, because in time the size of the region will be reduced by crossovers. The larger the shared area the closer the connection with a common predecessor from whom the gene originates [136]. The size of the shared regions allows IBD to be used in the genetic mapping of genes. Gruis et al. [137] described haplotype sharing in presumedly unrelated patients from a founder population. This sharing was determined by identity by descent of an area surrounding the disease gene. Houwen et al. [138] showed that mapping within 2 cM was possible using only three affected persons.

There are some points of importance which must be taken into consideration

when using IBD mapping. (1) The patients samples have to be collected in an established founder population. (2) For the assignment of haplotypes it is necessary to analyze also DNA of the parents. In case the phase is unknown it is possible to assign haplotypes according to their frequency. This, however, will reduce the power of the test drastically. (3) The allele frequency of the various markers used with IBD mapping has to be established in a control group within the same founder population.

A two step IBD approach can be used for gene mapping. In the first step a few not too far related patients with an identical genetic disorder can be haplotyped for a rough mapping of the disease gene. Markers can be selected with intervals of about 20 cM. This means that 100-200 markers will cover the whole genome. Fine mapping can be performed on the shared regions in a group of seemingly unrelated patients within a restricted area. These regions will be haplotyped then with highly polymorphic markers with relative distances of about 2 cM. This method allows fast and relative cheap gene mapping. A pooling strategy has been described [139] in which DNA from affected individuals were pooled and used as a PCR template for microsatellite primers. The amplification product of pooled DNA samples reflects all alleles represented in the pooled population. The relative frequency of the alleles correlates with the intensity of each allelic band in the gel after electrophoresis. In case of linkage disequilibrium of the microsatellite with a disease genotype there will be a shift in allele frequencies towards a homozygous allele pattern in the affected DNA pool. The use of the pooling strategy facilitates the identification of the disease loci by reducing the number of amplification products and gel lanes. Differences between control and affected pools can easily be examined, without the need of assigning individual genotypes.

IBD analysis can also be used for determination of the origins of certain mutations or the relationships of patients with identical genetic diseases. In case of cystic fibrosis, mutations have been reported which occur predominantly in certain countries [46]. The mutations may have originated in these countries not very long ago. An example is the A455E CF mutation, which is mainly detected in Southern-Holland and in Canada among certain French Canadians. It is likely that patients with this mutation have a common not too far predecessor. Chapter 6 describes the IBD analysis in Dutch and French Canadian patients.

References

1. Welsh MJ, Tsui L-C, Boat TF, Beaudet AL (1994) Cystic fibrosis In: Scriver CR, Beaudet AL, Sly WS et al.(eds) The Metabolic and Molecular Basis of Inherited Disease. New York, McGraw-Hill, pp 3799

2. Fanconi G, Uehlinger E, Knauer C (1936) The coeliac syndrome with congenital cystic pancreatic fibromatosis and bronchiectasis. Wien Med Wochenschr 86, 753

3. Andersen DH (1938) Cystic fibrosis of the pancreas and its relation to celiac disease: a clinical and pathological study. Am J Dis Child 56, 344

4. Farber S (1945) Some organic digestive disturbances in early life. J. Michigan Med Soc 44, 406

5. Anderson DH, Hodges RG (1946) Celiac syndrome. V. Genetics of cystic fibrosis of the pancreas with a consideration of etiology. Am J Dis Child 72, 62

6. Di Sant'Agnese PA, Darling RC, Perera GA, Shea E (1953) Abnormal electrolytic composition of sweat in cystic fibrosis of the pancreas. Clinical significance and relationship of the disease. Pediatrics 12, 549

7. Gibson LE, Cooke RE (1959) A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine by iontophoresis. Pediatrics 23, 545

8. Knowles M, Gatzy J, Boucher R (1981) Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. N Engl J Med 305, 1489

9. Quinton, PM (1983) Chloride impermeability in cystic fibrosis. Nature 301, 421

10. Tsui L-C, Buchwald M, Barker D, Braman JC, Knowlton R, Schumm JW, Eiberg H, Mohr J, Kennedy D, Plavsic N, Zsiga M, Markiewicz D, Akots G, Brown V, Helms C, Gravius T, Parker C, Rediker K and Donis-Keller H (1985) Cystic fibrosis locus defined by a genetically linked polymorphic DNA marker. Science 230, 1054

11. Estivill X, Scambler PJ, Wainwright BJ, Hawley K, Frederick P, Schwartz M, Baiget M, Kere J, Williamson R, Farrall M (1987) Patterns of polymorphism and linkage disequilibrium for cystic fibrosis. Genomics 1, 257

12. Rommens JM, Iannuzi MC, Kerem B, Drumm ML, Meimer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidaka N, Zsiga M, Buchwald M, Riordan JR, Tsui L-C, Collins FS (1989). Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 245, 1059

13. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui L-C (1989) Identification of the cystic fibrosis gene: genetic analysis. Science 245, 1073

14. Zielenski J, Rozmahel R, Bozon D, Kerem B-S, Grzelczak Z, Riordan JR, Rommens J, Chee L-C (1991). Genomic DNA sequence of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene. Genomics 10, 214

15. Cystic Fibrosis Genetic Analysis Consortium (December 16, 1994a) Cystic fibrosis mutation data (written communication)

16. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou J-L, Drumm ML, Iannuzzi MC, Collins FS, Tsui L-C (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245, 1066

17. Van der Hout AH, Van der Veen AY, Aten JA, Buys CHCM (1988) Localization of DNA probes with tight linkage to the cystic fibrosis locus by in situ hybridization using fibroblasts with a 7q22 deletion. Hum Genet 80, 161

18. Anderson MP, Rich DP, Gregory RJ, Smith AE, Welsh MN (1991a) Generation of cAMP-activated chloride currents by expression of CFTR. Science 251, 679

19. Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MN (1991b) Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. Science 253, 202

20. Anderson MP, Berger HA, Rich DP, Gregory RJ, Smith AE (1991c) Nucleotide triphosphates are required to open the CFTR chloride. Cell 67, 775

21. Bear CE, Duguay F, Naismith AL, Kartner N, Hanrahan JW, Riordan JR (1991) Cl^- channel activity in Xenopus oocytes expressing the cystic fibrosis gene. J Biol Chem 266, 19142

22. Berger HA, Anderson MP, Gregory RJ, Thompson S, Howard PW (1991) Identification and regulation of the cystic fibrosis transmembrane conductance regulator. J. Clin Invest 88, 1422

23. Dalemans W, Barbry P, Champigny G, Jallat S, Dott K, Dreyer D, Crystal RG, Pavirani A, Lecocq JP, Lazdunski M (1991) Altered chloride ion channel kinetics associated with the ΔF508 cystic fibrosis mutation. Nature 354, 526

24. Drumm ML, Wilkinson DJ, Smit LS, Worrell RT, Strong TV, Frizzel RA, Dawson DC, Collins FS (1991) Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes. Science 254, 1797

25. Kartner N, Hanrahan JW, Jensen TJ, Naismith AL, Sun S, Ackerley CA, Reyes EF, Tsui L-C, Rommens JM, Bear CE, Riordan JR (1991) Expression of the cystic fibrosis gene in non-epithelial invertebrate cells produces a regulated anion conductance. Cell 64, 681

26. Rommens JM, Dho S, Bear CE, Kartner N, Kennedy D, Riordan JR, Tsui L-C, Foskett JK (1991) cAMP-inducible chloride conductance in mouse fibroblast lines stably expressing the human cystic fibrosis transmembrane conductance regulator. Proc Natl Acad Sci USA 88, 7500

27. Tabcharani JA, Chang X-B, Riordan JR, Hanrahan JW (1991) Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. Nature 352, 628

28. Cliff WH, Schoumacher RA, Frizzell RA (1992) cAMP-activated Cl Channels in CFTR- transvected cystic fibrosis pancreatic epithelial cells. Am J Physiol 262, C1154

29. Cunningham SA, Worrell RT, Benos DJ, Frizzell RA (1992) cAMP-stimulated ion currents in Xenopus oocytes expressing CFTR cRNA. Am J Physiol 262, C783

30. Bijman J, Dalemans W, Kansen M, Keulemans J, Verbeek E, Hoogeveen A, De-Jong H, Wilke M, Dreyer D, Lecocq JP (1993) Low conductance chloride channels in IEC6 and CF nasal cells expressing CFTR. Am J Physiol 264, L229

31. Bijman J, Englert HC, Lang HJ, Greger R, Fromter E (1987) Characterisation of human sweat duct chloride conductance by chloride channel blockers. Pflugers Archiv Eur J Physiol 408, 511

32. Gregory RJ, Cheng SH, Rich DP, Marshall J, Paul S, Hehir K, Ostedgaard L, Klinger KW, Welsh MJ, Smith AE (1990) Expression and characterization of the cystic fibrosis transmembrane conductance regulator. Nature 347, 382

33. Halley DJJ, Bijman J, JongeHR de, Sinaasappel M, Neyens HJ, Niermeyer MF (1990) The cystic fibrosis defect approached from different angles. New perspectives on the gene, the chloride channel, diagnosis and therapy.Eur J Pediatr 149, 670

34. Rich DP, Anderson MP,Gregory RJ, Cheng SH, Paul S, Jefferson DM, McCann JD, Klinger KW, Smith AE, Welsh MJ (1990) Expression of cystic fibrosis transmembrane conductance regulator corrects Defective chloride channel regulation in cystic fibrosis airway epithelial cells.Nature 347, 358

35. Tsui L-C (1992) Mutations and sequence variations detected in the cystic fibrosis conductance regulator (CFTR) gene: a report from the Cystic Fibrosis Genetic Analysis Consortium.Hum Mut 1, 197

36. Welsh MJ, Smith AE (1993) Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 73, 1251

37. Bear CE, Li C, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR (1992) Purification and functional reconstruction of thecystic fibrosis trans membrane conductance regulator (CFTR).Cell 68, 809

38. Welsh MJ, Anderson MP, Rich DP, Berger HA, Denning GM, Ostedgaard LS, Sheppard DN, Cheng SH, Gregory RJ, Smith AE (1992).Neuron 8, 821

39. Collins FS (1992) Cystic fibrosis: molecular biology and therapeutic implications.Science 256, 774

40. Quinton PM (1990) Cystic fibrosis: a disease in electrolyte transport.FASEB J 4, 2709

41. Tsui L-C, Buchwald M (1991) In Advances HumGenet, H. Harris and K. Hirschhorn, eds (New York:Plenum Press), pp. 153

42. Gill DR, Hyde SC, Higgins CF, Valverde MA, Mintenig GM, Sepulveda FV (1992) Separation of drug transport and chloride channel functions of the human multidrug resistance P-glycoprotein.Cell 71, 23

43. Valverde MA, Diaz M, Sepulveda FV, Gill DR, Hyde SC, Higgins CF (1992) Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein.Nature 355, 830

44. Wine JL, Silverstein SC (1992) ATP and chloride conductance.Nature 360, 18

45. Gabriel SE, Clarke LL, Boucher RC, Stutts MJ (1993) CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship.Nature 263, 263

46. Cystic fibrosis Genetic Analysis Consortium (1994b) Population variation of common cystic fibrosis mutations. Hum Mut 4, 167

47. Delaney SJ, Rich DP, Thomason SA, Hargrave MR, Welsh MJ, Wainwright BJ (1993) CFTR splice variants are not conserved and fail to produce Clchannels. Nat Genet 4, 426

48. Strong TV, Wilkinson DJ, Mansoura MK, Devor DC, Henze K, Yang Y, Wilson JM, Cohn JA, Dawson DC, Frizzel RA, CollinsFS (1993) Expression of an abundant alternatively spliced form of the cystic fibrosis transmembrane conductance regulator (CFTR)gene is not associated with a cAMP-activated chloride conductance.Hum Mol Genet 2, 335

49. Cheng SH, Gregory RJ, MarshallJ, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE (1990) Defective intracellular transportand processing of CFTR is the molecular basis of most cystic fibrosis. Cell 63, 827

50. Thomas PJ, Shenbagamurthi P, Ysern X, Pedersen PL (1991) Cystic fibrosis transmembrane conductance regulator: nucleotide binding to a synthetic peptide. Science 251, 555

51. Sheppard DN, Rich DP, Ostedgaard LO, Gregory RJ, Smith AE, Welsh MJ (1993) Mutations in CFTR associated with mild-disease-form Cl- channels with altered pore properties. Nature 362, 160

52. Serre JL, Simon-Bouy B, Mornet E, Jaume-Roig B, Balassopoulou A, Schwartz M, Taillandier A, Boué J, Boué A (1990) Studies of RFLP closely linked to the cystic fibrosi locus throughout Europe lead to new considerations in populations genetics. Hum Genet 84, 449

53. Casals T, Vazquez C, Lazaro C, Girbau E, Gimenez FJ, Estivill X (1992) Cystic fibrosis in the Basque country: high frequency of mutation delF508 in patients of Basque origin. Am J Hum Genet 50, 404

54. Casals T, Nunes V, Palacio A, Giménez J, Gaona A, Ibáñez N, Morral N, Estivill X (1993) Cystic fibrosis in Spain: high frequency of mutation G542X in the Mediterranean coastal area. Hum Genet 91, 66

55. Morral N, Bertranpetit J, Estivill X, Nunes V, Casals T, Giménez J, Reis A, Varon-Mateeva R, Macek Jr M, Kalaydjieva L, Angelicheva D, Dancheva R, Romeo G, Russo MP, Garnerone S, Restagno G, Ferrari M, Magnani C, Claustres M, Desgeorges M, Schwartz M, Schwartz M, Novelli G, Ferec C, Arce M de, Nemeti M, Kere J, Anvret M, Dahl N, Kadasi L (1994) The origin of the major cystic fibrosis mutation (ΔF508) in European populations. Nature Genet 7, 169

56. Bertranpetit J, Cavalli-Sforza LL (1991) A genetic history of the Iberian Peninsula. Ann Hum Genet 55, 51

57. Tata F, Stanier P, Wicking C, Halford S, Kruyer H, Lench NJ, Scambler PJ, Hansen C, Braman JC, Williamson R, Wainwright BJ (1991) Cloning the mouse homolog of the human cystic fibrosis transmembrane conductance regulator gene. Genomics 10, 301

58. Snouwaert JN, Brigman KK, Latour AM, Malouf NN, Boucher RC, Smithies O, Koller BH (1992) An animal model for cystic fibrosis made by gene targeting. Science 257, 1083

59. Clarke LL, Grubb BR, Gabriel SE, Smithies O, Koller BH, Boucher RC (1992) Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. Science 257, 1125

60. Dorin JR, Dickinson P, Alton EWFW, Smith SN, Geddes DM, Stevenson BJ, Kimber WL, Fleming S, Clarke AR, Hooper ML, Anderson L, Beddington RSP, Porteous DJ (1992) cystic fibrosis in the mouse by targeted insertional mutagenesis. Nature 359, 211

61. Colledge WH, Ratcliff R, Foster D, Williamson R, Evans MJ (1992) Cystic fibrosis mouse with intestinal obstruction. Lancet 340, 680

62. Ratcliff R, Evans MJ, Cuthbert AW, MacVinish LJ, Foster D, Anderson JR, Colledge WH (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. Nature Genet 4, 35

63. O'Neal WK, Hasty P, McCray PB Jr, Casey B, Rivera-Pérez J, Welsh MJ, Beaudet AL, Bradley A (1993) A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. Hum Mol Genet 2, 1561

64. O'Neal WK, Beaudet AL (1994) Somatic gene therapy for cystic fibrosis. Hum Mol Genet 3, 1497

65. Johnson LG, Olsen JC, Sakardi B, Moore KL, Swanstrom R, Boucher RC (1992) Nature Genet 2, 21

66. Zabner J, Couture LA, Smith AE, Welsh MJ (1994) Correction of cAMP-stimulated fluid secretion in cystic fibrosis airway epithelia: Efficiency of adenovirus-mediated gene transfer in vitro. Hum Gene Ther 5, 585

67. Chu CS, Trapnell BC, Curristin SM, Cutting GR, Crystal RG (1992) Extensive posttranscriptional deletion of the coding sequences for part of nucleotide-binding fold 1 in respiratory epithelial mRna transcripts of the cystic fibrosis transmembrane conductance regulator gene is not associated with the clinical manifestations of cystic fibrosis. J Clin Invest 90, 785

68. Mulligan RC (1993) The basic science of gene therapy. Science 260, 926

69. Morgan RA, Anderson WF (1993) Human gene therapy. Annu Rev Biochem 62, 191

70. Houštěk J, Vávrová V (1962) On the incidence of cystic fibrosis of the pancreas in Czechoslovakia. Pediatr 17, 445

71. Simanková N, Vávrová V, Houštěk J (1964) Etude génétique de la mucuviscidose. Ann Génét 7, 84

72. Houštěk J, Vávrová V (1967) Notre experience à propos de la mucoviccidose. Rev Méd Liège 22, 421

73. Brunecky Z, Laxova R, Kamaryt J (1970a) Incidence and diagnosis of cystic fibrosis (mucoviscidosis): experiences with night sweat collection. Kinderärtzl Prax 38, 514

74. Brunecki Z, Simanková N, Vávrová V (1970b) Incidence and genetics of cystic fibrosis. Pediatr 25, 74

75. Brunecky Z (1972) The incidence and genetics of cystic fibrosis. J. Med Genetics 9, 33

76. Nielsen EL (1972) Cystic fibrosis: incidence in Danmark. Acta paediatr Scan 61, 377

77. Nielsen OH (1988) Cystic fibrosis in Denmark 1945 to 1985. Acta Paediatr Scan 77, 836

78. Carter CO, Watson GI (1967) General aspects of cystic fibrosis of the pancreas. Mod Probl Pediat 10, 372

79. Pugh RJ, Pickup JD (1967) Cystic fibrosis in the Leeds region: incidence and life expectancy. Arch Dis Child 42, 544

80. Hall BD, Simpkiss MJ (1968) Incidence of fibrocystic disease in Wessex. J. Med Genet 5, 262

81. Cain ARR, Deall AM, Noble TC (1972) Screening for cystic fibrosis by testing meconium for albumin. Arch Dis Childh 47, 131

82. British Paediatric Association Working party on cystic fibrosis (1988) Cystic fibrosis in the United Kingdom 1977-1985: an improving picture. Brit Med J 297, 1599

83. Nevanlinna HR (1972) The finnish population structure, a genetic and genealogical study. Hereditas 71, 195

84. Bernheim M, Monnet P, Jeune M, Robert JM, Comby J (1961) La maladie fibro-kystique des parenchymes glandulaires: Etude génétique de 41 familles. Pediatrie 16, 17

85. Gilly R, Hermier M, Robert JM, Challamel MJ, Gery G (1971) Genetic study of mucoviscidosis: statistics of 273 families. Arch franç Pédiat 28, 49

86. Gilly R (1973) Mucoviscidosis. Pathogenesis. Ann Pediat 20, 5

87. Feingold J, Hennequet A, Jehanne M, Feigelson J, Toudic L, Quinious O, Briard ML (1974) Incidence of pancreatic cystic fibrosis in France. Ann Génét 17, 257

88. Bois E, Feingold J, Demenais F, Runavot Y, Jehanne M, Toudic L (1978) Cluster of cystic fibrosis cases in a limited area of Brittany (France). Clin Genet 14, 73

89. Vivell VO, Jacobi H, Münchbach K (1963) Zur Mucoviscidosis im Kindesalter. Monatsschr Kinderheilkd 111: 62

90. Koch G, Schwanitz G (1968) Neuere human-genetische aspekte der mucoviscidose beim kind, in: Windorfer, A and Stephan, U (eds.), Mucoviscidose, 2. deutches symposion, Georg Thieme Verlag, Stuttgart.

91. Dietzsch HS, Gottschalk B (1975) CF screening: results of a pilot study on 10,000 newborns in the German Democratic Republic. European working group for cystic fibrosis. Dublin.

92. O'Reilly D, Murphy J, McLaughlin J, et al (1974). The prevalence of coeliac disease and cystic fibrosis in Ireland, Scotland and Wales. Int J Epidemiology 3, 247

93. Migliori V (1973) Mucoviscidosis. Minerva Med 64, 4906

94. Righetti ABB, Mighavacca M, Prampolini L, Guinta A (1976) Extensive neonatal screening of CF. In Proc VII Int Cystic Fibrosis Congress. Ed. Hennequet A, pp 153

95. Romeo G, Bianco M, Devoto M, Menozzi P, Mastella G, Giunta AM, Micalizzi C, Antonelli M, Battistini A, Santamaria F, Castello D, Marianelli A, Marchi AG, Manca A, Miamo A (1985) Incidence in Italy, genetic heterogeneity and segregation analysis of cystic fibrosis. Am J Hum Genet 37, 338

96. Ten Kate LP (1977) Cystic fibrosis in the Netherlands. Int J Epidem 6, 23

97. Clarke CA (1969) The relative fitness of human mutant genotypes, in: Roberts DF, Harrison GA (eds). Natural selection in human populations. Pergamon Press, London.

98. Nevin GB, Nevin NC, Redmond AD (1979) Cystic fibrosis in Northern Ireland. J. Med Genet 16, 122

99. Bozkowa K, Golebiowska H, Rutkowski J, Nowakowska A, Holzer J (1970) Incidence of cystic fibrosis in Poland. 1st ann Meeting Eur Working Group for Cystic Fibrosis. Stockholm, Sweden, August 24-25

100. Selander P (1962) The frequency of cystic fibrosis of the pancreas in Sweden. Acta paediatr 51, 65

101. Kollberg H (1970) Cystic fibrosis in Sweden, 1st Ann Meeting Eur Working Group for Cystic Fibrosis. Stockholm, Sweden.

102. Kollberg H (1976) Screening for cystic fibrosis (CF) by analysis of albumin in meconium. VII. int. Cystic Fibrosis congress, May 31-June 1-3. Paris, France, pp. 32

103. Kollberg H (1982) Incidence and survival curves of cystic fibrosis in Sweden. Acta Paediatr Scan 71, 197

104. Kaiser D, Morer HP, Hammershlag P (1976) Experience of 3 years meconium screening in Switzerland. In Proc VII Int Cystic Fibrosis Congress. Ed. Hennequet A, pp 148

105. Gürson CT, Sertel H, Gürkan M, Pala S (1973) Newborn screening for cystic fibrosis with the chloride electrode and neutron activation analysis. Helvetica paediatrica acta 28, 165

106. Wright SW, Norton NE (1968) Genetic studies on cystic fibrosis in Hawaii. Am J Hum Genet 20, 157

107. Goodman HO, Reed SC (1952) Heredity of fibrosis of the pancreas: possible mutation rate of the gene. Am J Hum Genet 4, 59

108. Kitzis A et al. (1988) Unusual segregation of cystic fibrosis alleles. Nature 336, 316

109. Romeo G, Devoto M, Galietta LJV (1989) Why is the cystic fibrosis gene so frequent? Hum Genet 84, 1

110. Danks DM, Allan J, Anderson CM (1965) A genetic study of fibrocystic disease of the pancreas. Ann Hum Genet London 28, 323

111. Super M, Shalkwyk DJ van (1979) Heterozygote advantage in cystic fibrosis: mosquito test. Clin Genet 16, 65

112. Cassano WF (1985) Cystic fibrosis and the plague. Med Hypoth 18, 51

113. Jorde LB, Lathrop GM (1988) A test of the heterozygote advantage hypothesis in cystic fibrosis carriers. Am J Hum Genet 42, 808

114. Knudson AG, Wayne L, Hallet WY (1967) On the selective advantage of cystic fibrosis heterozygotes. Am J Hum Genet 19, 388

115. Gloria-Bottini F, Antonelli M, Quattrucci S et al. (1980) Is there a relationship between sex of cystic fibrosis carriers and sex ratio of their offspring? Hum Genet 54, 79

116. Shier WT (1979) Increased resistance to influenza as a possible source of heterozygote advantage in Cystic fibrosis. Med Hypoth 5, 661

117. Meindl, RS (1987) A selective advantage for cystic fibrosis heterozygotes. Am J Phys Anthropol 74, 39

118. Hollander DJ (1982) Etiogenesis of the european cystic fibrosis polymorphism: heterozygote advantage against venereal syphilis? Med Hypoth 8, 191

119. Baxter PS, Goldhill J, Hardcastle J, Hardcastle PT, Taylor CJ (1988) Accounting for cystic fibrosis. Nature 336, 211

120. Rodman DM, Zamudio S (1991) The cystic fibrosis heterozygote advantage surviving cholera? Med Hypoth 36, 253

121. Bijman J, De Jonge H, Wine J (1988) Cystic fibrosis advantage. Nature 336, 430

122. Hansson GC (1988) Cystic fibrosis and chloride-secreting diarrhoea. Nature 333, 711

123. Gabriel SE, Brigman KN, Koller BH, Boucher RC, Stutts MJ (1994) Cystic fibrosis heterozygote resistance to cholera toxin in the cystic fibrosis mouse model. Science 266, 107

124. Wilfond BS, Fost N (1990) The cystic fibrosis gene: Medical and social implications for heterozygote detection. JAMA 263, 2777

125. Ten Kate LP, Tijmstra TJ (1989) Carrier screening for cystic fibrosis. Lancet, 973

126. Kaplan F, Clow C, Scriver CR (1991) Cystic fibrosis carrier screening by DNA analysis: A pilot study of attitudes among participants. Am J Hum Genet 49, 220

127. Watson EK, Marchant J, Bush A, Williamson B (1992) Attitudes towards prenatal diagnosis and carrier screening for cystic fibrosis among the parents of patients in a paediatric cystic fibrosis clinic. J Med Genet 29, 490

128. Williamson R (1993) Universal community carrier screening for cystic fibrosis? Nature Genet 51, 943

129. Cuppens H, Marymen P, DeBoeck C, Cassiman JJ (1993) Detection of 98.5 % of the mutations in 200 Belgian cystic fibrosis alleles by reverse dot-blot and sequencing on the complete coding region and exon/intron junctions of the CFTR gene. Genomics 18, 693

130. Férec C, Audrézet MP, Mercier B, Guillermit H, Moullier P, Quéré I, Verlinque C (1992) Systematic Screening for mutations in the cystic fibrosis gene: New implications for carrier detection. Nature Genet 1, 188

131. Cheadle JP, Goodchild MC, Meredith AL (1993) Direct sequencing of the complete CFTR gene: The molecular characterzation of 99.5 % of CF chromosomes in Wales. Hum Mol Genet 2, 1551

132. Shuber AP, Skoletsky J, Stern R, Handelin BL (1993) Efficient 12-mutation testing in the CFTR gene: A general model for complex mutation analysis. Hum Mol Genet 2, 153

133. Ferrie RM, Schwartz MJ, Robertson NH, Vaudin S, Super M, Malone G, Little S (1992) Development, multiplexing, and application of ARMS test for common mutations in the CFTR gene. Am J Hum Genet 51, 251

134. Syvänen A-C, Landegren U (1994) Detection of point mutations by solid-phase methods. Hum Mutat 3, 172

135. Beaudet AL, O'Brien WE (1992) Advantages of a two-step laboratory approach for cystic fibrosis carrier screening. Am J Hum Genet 50, 439

136. Te Meerman GJ, Meulen MA van der, Sandkuijl LA (1995) Perspectives of identity by descent (IBD) mapping in founder populations. (Submitted)

137. Gruis N, Sandkuijl, LA, Bergman W, Frants RR (1994) Common 9p Haplotype in Dutch Famm m families, PhD thesis N. Gruis, The genetics of the familial atypical multiple melanoma syndrome, Leiden

138. Houwen RMJ, Baharbo S, Blankenship K, Raeymaekers P, Juyn J, Sandkuyl LA, Freimer NB (1994) Genome screening by searching for shared segments: mapping a gene for benign recurrent intrahepatic cholestasis. Nature Genet 8, 380

139. Carmi R, Rokhlina T, Kwitek-Black AE, Elbedour K, Nishimura D, Stone EM, Sheffield VC (1995) Use of a DNA pooling strategy to identify a human obesity syndrome locus on chromosome 15. Hum Mol Genet 4, 9